

IMMUNOFLUORESCENT STAINING: THE FLUORESCENT ANTIBODY METHOD¹

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I. PRINCIPLES OF IMMUNOFLUORESCENT STAINING

Methods employing fluorescent antibodies are either the direct or indirect product of Albert Coons' investigations. They are referred to here as immunofluorescent staining or IF staining. In essence, IF staining consists of labeling antibodies with a fluorescent dye, allowing the labeled antibodies to react with their specific antigen, and observing the reaction product under the fluorescence microscope. The latter consists of a standard microscope with a dark-field condenser and an ultraviolet light source. Since Coons *et al.* (30) introduced the method in 1942, over 300 reports have appeared. IF staining has added new dimensions to our understanding of such subjects as antibody synthesis, viral infection, and cellular localization of tissue antibodies. Several reviews of the subject are available (23-26, 28, 29, 113). The objectives of this review are: (a) to summarize the principles of staining with fluorescent antibodies, particularly as they have evolved since Coons' review of 1956 (24); (b) to sum-

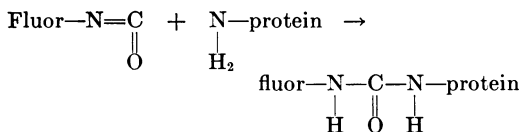
marize the major original contributions made by this method since 1956 (older papers are reviewed only as they relate to the more recent developments); (c) to relate immunofluorescence to the classic concepts of immunology.

A. Fluorescent Antibody Labeling

Compounds which emit light in one wave length range when illuminated by light of a shorter wave length range are fluorescent. Most fluorescent compounds that have been studied are excited by light in the ultraviolet or near ultraviolet region and emit light in the visible range. Fluorescent dyes are called fluorochromes.

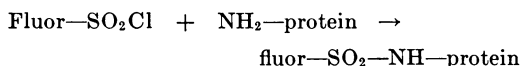
The coupling of fluorochromes to antibodies forms the basis of staining with labeled antibody. Briefly, three methods of coupling fluorescent dyes to proteins have been utilized to prepare conjugates for IF staining. (As used here the term "conjugates" refers to fluorochrome-labeled proteins.) According to the original procedure of Coons, Creech, Jones, and Berliner (30) an isocyanate derivative of fluorescein is prepared by reaction of amino group of the fluor with phosphine. The isocyanate reacts with free amino groups of proteins to form a carbamido linkage:

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Later, Riggs *et al.* (135) introduced a procedure for conjugating isothiocyanate derivatives of fluorescent dyes (fluor—N=C=S) with proteins. Presumably the reaction with proteins is similar to that of isocyanates.

Conjugates may also be prepared with sulfonyl chloride derivatives of fluorochromes. This method had been used by Weber (155) and others to label proteins with the fluorochrome 1-dimethyl naphthalene. Though useful as a protein label, this fluorochrome is of limited value in IF staining because the intensity of its fluorescence is low. According to data reported by Uehleke (151), the emission intensity of 1-dimethyl-5-naphthalene sulfonic acid when excited by light at 365 mμ is about one-third that of fluorescein. Conjugation with sulfonyl chloride derivatives occurs through free amino groups of the protein.



According to Uehleke (152), sulfonyl chlorides also react with secondary amines and alcohol groups on proteins.

The two fluorochromes used most widely thus far are fluorescein and rhodamine. Fluorescein has a yellowish green fluorescence with a maximum at about 550 mμ (48, 151), and lissamine rhodamine B 200 or rhodamine B has a reddish orange fluorescence with a maximum at about 640 mμ (151). These have been the fluorochromes of choice because the intensity or efficiency of fluorescence of these compounds is greater than those of other fluorochromes that have been studied. The green fluorescence of fluorescein offers two important advantages over the red fluorescence of rhodamine: (a) although fluorescein and rhodamine have about the same intensity of fluorescence, the eye is more sensitive to apple green than to reddish orange; (b) red autofluorescence is more common in nature than green autofluorescence.

Conjugation of both fluorescein and rhodamine with proteins has been carried out by the three methods described above. According to the original procedure of Coons *et al.* (30), fluorescein

isocyanate is conjugated to antibody containing serum globulin. Hiramoto, Engel, and Pressman (77) describe a procedure for conjugating tetramethylrhodamine to antibody globulins by an isocyanate linkage. Riggs *et al.* (135) introduced procedures for conjugation of fluorescein and rhodamine derivatives to globulin with isothiocyanate linkages. Isothiocyanate derivatives have the advantage of being more stable and readily available. J. D. Marshall, Eveland, and Smith (108) report a fourfold greater sensitivity of fluorescent reactions with fluorescein isothiocyanate than with fluorescein isocyanate, although this report has not yet been confirmed. At present these are the most widely used labeling reagents. Chadwick, McEntegart, and Nairn (16) and Uehleke (151) described methods for conjugating rhodamine derivatives to proteins with sulfonyl chloride linkages. The ratio of rhodamine to protein in the procedure of Uehleke is higher; however, Chadwick *et al.* report on staining with their conjugates, whereas Uehleke does not. Preliminary experiments in the writer's laboratory indicate that the molar ratio of rhodamine to protein is about 2:1 in conjugates prepared according to the procedure of Chadwick, McEntegart, and Nairn. Uehleke also describes a procedure for preparing a sulfonyl chloride derivative of fluorescein and for preparing a protein conjugate with it. Although staining reactions were not carried out with this conjugate, the work is of interest because conjugation with sulfonyl chloride derivatives is more rapid than conjugation with isocyanate or isothiocyanate derivatives (16). A promising fluorochrome is 3-hydroxypyren-5,8,10-trisulfonic acid. According to Uehleke (151) the wave length of its fluorescent emission maximum (530 mμ) is similar to that of fluorescein, but its emission intensity is three times greater than that of fluorescein. This observation has not as yet been confirmed. Trials of IF staining with conjugates of hydroxypyren are indicated.

Serum globulins for conjugation may be prepared by fractionating serum with 50 per cent saturated ammonium sulfate (*e.g.*, 30) or 23 per cent sodium sulfate (*e.g.*, 80) or by the cold alcohol method of Cohn *et al.* (21). Chadwick, McEntegart, and Nairn (16) prepared conjugates for IF staining from whole serum.

Some noteworthy modifications in the procedure of conjugating isocyanate and isothio-

cyanate derivatives of fluorochromes to serum globulins have been introduced. According to the procedure of Coons (26), serum globulins are mixed with acetone, dioxane, and carbonate buffer (pH 9) before fluorescein isocyanate is added. Goldman and Carver (67) conjugated globulins without the addition of acetone and dioxane. Fluorescein isocyanate solution was dried on filter paper strips and stored in a desiccator. Buffered serum globulins are conjugated simply by adding the paper impregnated with fluorescein isocyanate and shaking. Marshall, Eveland, and Smith (108) reported on the conjugation with fluorescein isocyanate and isothiocyanate with and without the addition of dioxane and acetone. According to their data, conjugates prepared without the addition of organic solvents have greater activity.

Although antibodies labeled with fluorescein remain serologically active (30), some slight changes in the properties of labeled proteins are manifest. The disposition *in vivo* of labeled egg albumin is not quite the same as that of the unlabeled egg albumin. Mayersbach and Pearse (112) called attention to a discrepancy between the renal localization of unlabeled proteins as reported by Coons, Leduc, and Kaplan (33) and the localization of fluorescein-labeled bovine serum albumin reported by Schiller, Schayer, and Hess (139). Coons *et al.* reported demonstration of egg albumin, bovine serum albumin, and human γ -globulin both on and in nuclei of renal tubular epithelium and other cells, whereas Schiller *et al.* reported localization of fluorescein-labeled bovine serum albumin only on the surface of the nuclear membrane. Mayersbach and Pearse confirmed both observations with the use of labeled and unlabeled egg albumin. The nuclear membrane is impermeable to the fluorescein-labeled protein but is permeable to the unlabeled protein. Another type of change resulting from conjugation was demonstrated by Curtain (38). The electrophoretic mobility of fluorescein-labeled γ_2 -globulin is greater than that of unlabeled globulin. Uehleke (152) also reported increased mobility of proteins conjugated with other fluorochromes.

B. Immunofluorescent Staining Procedures

Authoritative and detailed discussions of fluorescent antibody staining techniques may be found in Coons' review of the subject (26) as well as in various reports on observations made

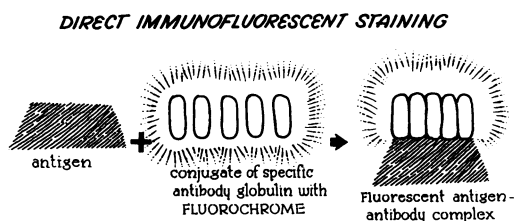


Figure 1. Outline of direct IF staining. This reaction sequence is usually carried out *in vitro*, sometimes *in vivo*.

by this method. The object here is to summarize the concepts that have been evolved and applied in fluorescent antibody studies. Antibody reactions have been localized in sections of tissue (31), smears of cells (*e.g.*, 119), smears of antigens (*e.g.*, 56), tissue blocks which are sectioned after treatment with labeled antibodies (110), cell suspensions (*e.g.*, 8), and living animals (*e.g.*, 60). Several immunologic reaction sequences have been successfully utilized.

1. Coons' original procedure (30) consisted of applying fluorescein-labeled solutions of antibody directly to preparations containing the antigen(s). This procedure is referred to as direct immunofluorescent staining (or direct IF staining.) The procedure is illustrated in figure 1.

2. Indirect staining with fluorescent antibody is carried out by treating the preparation containing the antigen first with unlabeled specific antiserum. The resulting antigen-antibody complex is rendered visible by treating it with a fluorescent-labeled antibody to the specific (antibody) globulin. For example, specific rabbit antibodies may be localized with labeled goat or horse antirabbit γ -globulin antibodies; specific human antibodies may be localized with conjugates of rabbit or goat antihuman γ -globulin antibodies, and so on. This procedure is referred to as *indirect IF staining*. It is illustrated in figure 2. Weller and Coons (158) were the first to describe the procedure. Watson, in Coons' laboratory was the first to demonstrate it. *Indirect IF staining* makes it possible to use a single conjugate to study a variety of antisera of a given species. The procedure is more readily controlled and more sensitive than direct IF staining. Indirect IF staining has greatly expanded the utilization of the fluorescent antibody method. Conjugates of anti-globulins are now commercially available.

3. A complement-staining procedure was reported by Goldwasser and Shepard (71). This

INDIRECT IMMUNOFLUORESCENT STAINING

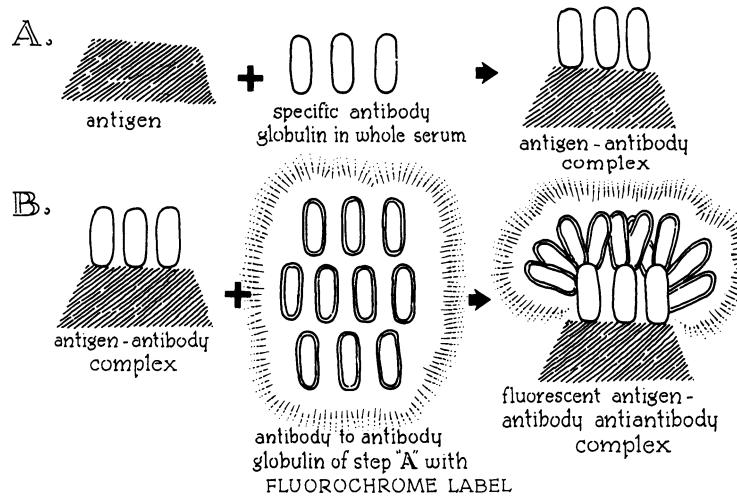


Figure 2. Outline of *indirect* IF staining. The second step with the labeled antiglobulin (e.g., labeled rabbit antibodies to human γ -globulin for demonstration of human antibodies) is carried out *in vitro*.

technique is based on the fact that complement, if present, participates in immunologic reactions. By heating a serum to 56 C for 30 minutes its complement activity is destroyed. Guinea pig serum is rich in complement and is usually used as a source of complement in serologic reactions. The procedure consists of applying a mixture of a specific antiserum and whole fresh guinea pig serum to a section or smear containing the antigen. The complex of antigen, antibody, and complement which forms on the slide is visualized by applying fluorescent-labeled antibodies to guinea pig complement. Goldwasser and Shepard report greater sensitivity in detecting serologic reactions by complement staining than by indirect IF staining. Klein and Burkholder (95) also report specific IF staining with fluorescein-labeled anti-complement antibodies.

Triple layer staining has been reported, though the results are open to question. In our experience, nonspecific staining is excessive. Still other IF staining procedures have been applied in studies of antibody localization. These are summarized in the section on antibody synthesis and reactions *in vivo*.

C. Controls

In staining with labeled antibodies, as in any serologic method, controls constitute an integral part of experimental procedures. Three types of

controls should be applied. (a) In all IF experiments it is necessary to *identify the observed fluorescence as a serologic reaction*. The precise nature of the necessary controls depends to some extent on the type of IF staining. A list of appropriate controls for direct IF staining is detailed by Coons (26). These include: inhibition of reaction by adsorption of conjugate with specific antigen and failure to inhibit by adsorption with heterologous antigen; inhibition of staining by pretreatment with unlabeled antiserum or by mixing labeled with unlabeled antiserum; and negative reactions with labeled normal serum globulin or heterologous antiserum globulin. Each experiment carried out by indirect IF staining should include control preparations treated with conjugate alone, as well as preparations treated with normal serum or heterologous antiserum (in place of the specific antiserum) and antiglobulin conjugate. Still other controls of the serologic nature of staining reactions are suggested in the literature on IF studies. Immunofluorescent staining by its very nature frequently provides intrinsic evidence of the serologic nature of staining reactions, e.g., staining of selected bacteria in a mixed population or staining of specific histologic structures in tissue sections with tissue antibodies. (b) In any system that has not already been thoroughly investigated by immunofluorescent methods, it is necessary to *demonstrate the*

serologic specificity of the observed reaction. That is, the spectrum of cross reactions (if any) of the antibodies with related or different antigens must be ascertained. The serologic specificity of antibodies that have already been studied for cross reactions by other serologic methods should be reinvestigated by IF staining, since different spectra of cross reactions are observed by different methods. For example, antibodies to group A β -hemolytic streptococci cross react with group C by IF staining (118) but not by precipitation. As a rule, the number of control antigens that should be tested is one more than the number already tested. (c) Ultimate confirmation of IF staining reactions lies in the *demonstration of the given reaction by other serologic methods.* Such confirmation is always desirable.

D. Nonspecific Staining

Most fluorescent antibody studies are complicated by nonspecific staining of the antigen with the labeled globulins. Some recent studies shed light on the nature of these nonspecific staining reactions. Curtain (38) fractionated fluorescein-labeled antisera by electrophoresis convection. It is evident from his comparison of antibody titers, nonspecific staining reactions, and protein concentration of the fractions, that serologically inactive as well as active fractions give rise to nonspecific staining. Louis, Hughes, and their associates (see 106 for references) report that a variety of fluorescein- and rhodamine-labeled proteins, including normal serum globulins and albumins as well as labeled egg albumin, give rise to nonspecific staining. The prime interest of these investigators is the differentiation of normal and neoplastic cells by their staining with labeled proteins. Louis (106) summarizes the histologic distribution of these nonimmunologic staining reactions with an extensive list of normal tissues which are stained and neoplastic tissues which do not stain. The cytoplasm of all normal epithelial cells and white blood cells in mammalian tissues stained, whereas connective tissue, central nervous system tissue, and red blood cells did not.

Adsorption of conjugates with acetone-dried liver powders according to the method of Coons, Leduc, and Connolly (32) is one of the most widely used methods for removing nonspecific staining. Frequently, only partial removal of nonspecific staining is effected by this method (*e.g.*, 38). Mayersbach (111) notes that freeze

drying, storage of frozen sections, formalin fixation, and pretreatment of tissue with alkaline buffers reduce nonspecific staining. In my experience, pretreatment with alkaline buffers or formalin not only inhibits nonspecific staining but also destroys certain antigens in epithelial cells.

Observations such as those of Louis (*e.g.*, 106) suggest that normal serum proteins bind with a variety of antigens. This is also manifest in controls of indirect IF stains. Control preparations treated with normal serum and conjugate are consistently stained more strongly than preparations treated with only conjugates. Thus, Smith, Marshall, and Eveland (143) render nonspecific staining red or brown by pretreating preparations with rhodamine conjugates of normal serum globulin. Similarly, in studies with indirect IF staining, nonspecific reactions are inhibited by pretreatment of preparations with normal serum of another species (*e.g.*, 81). Another concept which has been applied in the writer's laboratory is that nonspecific binding of serum proteins is different from binding of specific serologic reactions. Thus extraction of preparations with 50 per cent glycerol has been used to remove nonspecific staining.

Nonspecific staining reactions are probably in part a function of the amount of fluorescein derivative used in the conjugation mixture. Coons and his associates (30, 31) obtained optimal results with 5 mg of fluorescein isocyanate (measured as fluorescein amine) per 100 mg of crude globulin protein. Ratios of molecular fluorescein to protein of about 2:1 are obtained in such conjugates. Conjugates with lower ratios of fluorescein to protein yield progressively less IF staining. There is little information in the literature relevant to the influence of larger amounts of fluor in the conjugation mixture on nonspecific and specific staining. Available information suggests that nonspecific staining is increased, whereas specific staining remains the same or decreases. This increased nonspecific staining appears to be caused by the increased affinity of the altered proteins for a variety of substances.

Two extreme examples serve to illustrate the type of situations that may be encountered. At one extreme are leukocytes. Coons *et al.* (32), Louis (106), and others (40) who have encountered them in fluorescent antibody studies have noted their affinity for nonspecific staining. No

amount of adsorption of conjugates will remove this nonimmunologic staining reaction. Danaher, Friou, and Finch (40) reported on indirect IF staining of human white blood cells with isoantibodies. They were able to observe some specific staining despite the strong nonspecific reaction. Indirect immunofluorescent staining with isoantibodies or autoantibodies is further complicated by the fact that the conjugate reacts directly with γ -globulin and related antigens in the tissue as well as specific antibodies. Thus Danaher *et al.* report observations which suggest that γ -globulin is present in or on normal leukocytes. In sharp contrast to the nonspecific staining problems encountered with white cells is the total lack of nonspecific staining of red blood cells. Cohen, Zuelzer, and Evans (19) report completely specific staining of red cells with labeled blood group isoantibodies *without* adsorption of conjugates. These authors report detailed observations on another type of nonspecific staining frequently observed: the trapping of conjugate under the antigen on the slide. Trapped conjugate, as these authors point out, can be readily distinguished from specific staining.

II. ANTIBODY SYNTHESIS AND REACTIONS IN VIVO

A. Synthesis

The first direct evidence for the cellular site of antibody formation came from studies of Coons *et al.* (32). Coons' review of 1956 (24) should be consulted for an authoritative discussion. Basically, four types of fluorescent antibody procedures have been brought to bear on this problem. Two of them yielded valuable results. The procedures and the essential results obtained with them may be summarized here.

1. Historically, the first approach used by Coons was direct staining for antigen *e.g.* (22). The globulin fractions of antisera to the tracer antigen were labeled. Animals treated by injection with the tracer antigen were killed, and the localization of the antigen was determined by direct staining. By this method, a clear demonstration of antigen localization was achieved. However, it was manifest from the studies of Coons and his associates that the cellular localization of antigen and the site of antibody synthesis are not the same.

2. The next logical step is to label *antigens* with fluorescein. Information on antibody syn-

thesis has not been obtained by this method. However, Germuth *et al.* (60) and others have studied antibody reactions *in vivo* by this method. It should be pointed out that not all antigens can be labeled with fluorescein by the methods used for labeling globulins.

3. The most significant results have been achieved by a two-step reaction (2, 27, 32, 123, 160, 164). Sections of tissue containing antibody are flooded first with the antigen, then with labeled antibodies to this antigen (see figure 3). It is by this method that the first direct demonstration of antibody inside of plasma cells was achieved (32) and the long standing controversy concerning the site of antibody synthesis was finally resolved.

4. More recently, direct IF staining for γ -globulin with labeled antiglobulins has been utilized to study antibody synthesis (127, 130). Results are in essential agreement with those obtained by the two-step method described above. However, direct staining for γ -globulin is a relatively nonspecific histochemical approach. The specificity of detected antibodies is unknown. Indeed, this method assumes that all γ -globulins are antibodies.

The observations on antibody synthesis in the cytoplasm of the lymphocyte-plasma cell series by Coons *et al.* (32) have been confirmed and extended in several laboratories (2, 130, 160, 164). White (160) noted that Russell bodies within plasma cells are composed largely of antibodies. Witmer (164) studied antibody formation in the eye by the double layer technique. Plasma cells which infiltrated the iris and ciliary body of the eye during inflammatory conditions were demonstrated to contain antibodies. Antibody titers as determined by tanned cell hemagglutination roughly paralleled the appearance of antibody-containing plasma cells. Askonas and White (2) examined for formation of antibody to ovalbumin in guinea pigs. They correlated the number of plasma cells stained by the fluorescent antibody method with the amount of anti-ovalbumin antibody produced *in vitro* in various tissues. With the exception of the bone marrow, the correlation was good.

More recently, Coons (27) reported demonstration of occasional cells with intranuclear antibody. Such nuclei were observed to contain bright granules with high antibody concentrations. These granules are interpreted to be nucleoli. He further reported that a large number of

**DOUBLE LAYER IMMUNOFLUORESCENT STAINING
FOR ANTIBODY LOCALIZATION**

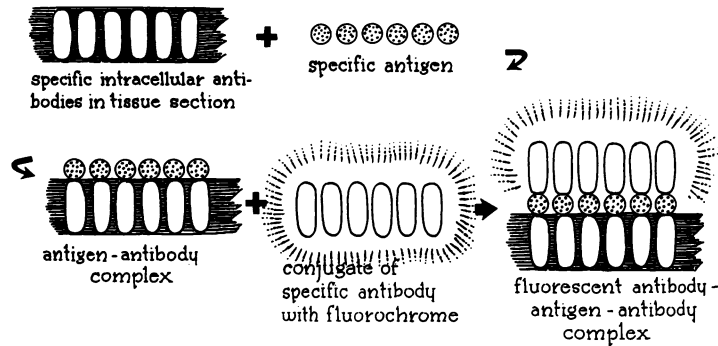


Figure 3. Procedure for indirect fluorescent antibody staining for specific antibodies in tissue

nucleoli are found in association with active antibody synthesis. The passive transfer of antibody-producing cells has been studied intensively by Dixon and his associates (46, 137, 138). They injected lymphatic cells (lymph node cells, thymus cells, and peritoneal exudates) from immunized animals into recipient animals which had been rendered immunologically inert by whole body irradiation. In a recent publication, Neil and Dixon (123) utilized the fluorescent antibody method together with other histologic methods to trace the fate of cells producing antibody after their transfer into recipient animals. Their data suggest that lymphocytes from donors are transformed into plasma cells; that the development of plasma cells corresponds to the appearance of cells containing antibody; and that secondary or anamnestic responses can be elicited in animals passively sensitized with cells of the lymphocyte series. In all these studies, the cellular localization of antibodies was carried out by the two-step staining method. (See figure 3 and procedure 3 above.)

Since many antibodies are γ -globulins, intracellular antibody localization can also be achieved by direct IF staining for γ -globulin. (See procedure 4 above.) Some noteworthy studies based on the assumption that all γ -globulin molecules are antibodies have been reported. Studies of γ -globulin localization in human lymph nodes and spleen were carried out by Ortega and Mellors (130). Specific γ -globulin localization is reported in plasma cells with and without Russell bodies, and in a cell type referred to as "intrinsic cells of germinal centers" of lymph nodes. According to

the authors, these are exactly the same cell types that were demonstrated by Coons *et al.* to produce specific antibodies. Localization of γ -globulin in the spleen and liver of patients with hyperglobulinemia was studied by Ohta *et al.* (127). According to their report, γ -globulin was found in plasma cells and related cell types as well as in large mononuclear splenic tumor cells.

B. Hypersensitivity Reactions

The mechanism of hypersensitivity reactions induced by reactions of circulating antibodies *in vivo* has been elucidated by immunofluorescent staining (17, 45, 60, 107, 114, 127, 142, 165). The approaches used in these studies include direct IF staining for antigen and/or γ -globulin on sections of hypersensitivity lesions and the injection of fluorescein-labeled antigens into sensitized animals, followed by observations on the localization of the labeled antigen in lesions. Many investigators prefer to support IF staining observations with other serologic studies. Among the classic experimental hypersensitivity reactions that have been studied with the aid of staining with labeled antibody are: local arthus reactions (local antibody-induced lesions), anaphylactic reactions (immediate systemic hypersensitivity reactions in a host previously sensitized to the offending antigen), serum sickness (systemic hypersensitivity reactions to foreign serum proteins which usually develop 5 to 10 days after injection), glomerulonephritis, and nephrotoxic serum-induced kidney disease.

Germuth *et al.* (60) demonstrated local Arthus reactions in the avascular cornea of the eye.

Reactions were produced by injecting fluorescein-labeled bovine serum albumin (BSA) into the center of the cornea of rabbits previously sensitized to BSA. Lesions took the form of an opaque arc around the site of inoculation. By examination with ultraviolet light, the arc was demonstrated to correspond precisely to the localization of the labeled antigen. Passive sensitization was demonstrated by injecting into normal rabbit cornea antisera to BSA on one side and fluorescein-labeled BSA on the other. An opaque line developed between injection sites, again corresponding to the antigen localization. These observations leave little doubt that antigen-antibody complexes are directly associated with the observed lesions of the Arthus reaction. The pulmonary thrombi observed in active or passive anaphylaxis in rabbits were identified by McKinnon *et al.* (107) as antigen-antibody precipitate. The identity of these thrombi with immune precipitate was demonstrated by labeling the challenging antigen BSA or bovine γ -globulin (BGG) with fluorescein and observing the specific fluorescence of the immune precipitate. The authors suggest an association between these thrombi and the pathogenesis of anaphylaxis in the rabbit. Dixon *et al.* (45) investigated serum sickness reactions by injecting into rabbits antigens (BSA and BGG) labeled with radioactive iodine and fluorescein. Careful kinetic studies on the disappearance of antigen and the development of antibodies clearly indicated that characteristic serum sickness lesions in the kidneys and arteries appear during the time interval in which both antigen and antibody are present. Specific localization of antigen and host γ -globulin at the site of the lesions was demonstrated by appropriate direct IF staining for BSA and rabbit γ -globulin.

Glomerulonephritis is a prominent feature of the serum sickness syndrome. This is in part a reflection of the fact that all manner of foreign antigens tend to become localized in the kidney. Kaplan's (89) fluorescent antibody studies on the distribution of polysaccharides of group A β -hemolytic streptococci illustrate this fact. The observed sequellae are localization of host γ -globulin and a local Arthus reaction. Some investigators have focused their attention solely on the kidney lesions following foreign antigen injection. Direct association of localization of host γ -globulin in the kidney with BSA-induced glomerulonephritis (in rabbits) was demonstrated

in fluorescent antibody studies of Mellors *et al.* (114). In effect, this is part of the serum sickness syndrome. Bovine serum proteins are not unique in their capacity to induce the development of glomerulonephritis. A variety of other animal proteins and bacterial antigens have been used to induce similar disease processes. Wood and White (165) studied glomerulonephritis produced in mice by repeated injections of heat-killed suspensions of *Proteus mirabilis*. The bacterial antigen was demonstrated by direct IF staining to be associated with kidney lesions. Agglutination titers with *P. mirabilis* appeared to be related to the severity of the kidney lesions. Efforts to extrapolate observations to the pathogenesis of human kidney diseases and other diseases have been reported from several laboratories, chiefly from the laboratories of Mellors (115), Dixon (44), and Gitlin (62). Localization of γ -globulin and other serum proteins in the kidney lesions and other pathologic tissues has been investigated. These important studies are ably reviewed by Mellors (113). Such studies are for the most part *histochemical* in nature, since the immunologic basis of the observed localization of γ -globulin and other serum proteins is not clearly understood.

More specific localization of foreign antigens in the kidney is achieved with nephrotoxic antisera. These are usually antisera to the kidney of the animal to which the serum was administered; *e.g.*, rabbit antisera to rat kidney injected into the rat produce nephrotoxic nephritis. The disease is chronic and sometimes fatal. The incidence of kidney lesions following injection of nephrotoxic antisera is reported to be 100 per cent. However, the incidence of kidney lesions is reported to be as high as 80 per cent following the injection of large doses of other foreign antigens (114). Thus, as Ortega and Mellors point out (129), the consistent appearance of kidney lesions following injection of nephrotoxic sera is simply the result of the more specific kidney localization of the antibodies. This interpretation is consistent with the views of Kay (91). His data suggested that kidney lesions and death in nephrotoxic nephritis resulted not from the action of the nephrotoxic sera on the kidney, but from the reactions of the host antibodies to the nephrotoxic antibodies in the kidney. It is noteworthy that not only kidney antisera but also antisera to lung and other organs are effective in producing kidney lesions (Baxter and Goodman (5)). This

is a reflection of the fact that antibodies to kidney are, in large part, not organ specific but species specific. That is, they react with other organs and tissues of the antigen-supplying species. This was also demonstrated by localization *in vivo* and *in vitro* of anti-kidney antibodies (Hiramoto *et al.*, 79). Localization *in vivo* was demonstrated by intravenous injection of rabbit anti-rat kidney antiserum into rats and staining rat organ sections with anti-rabbit serum globulin conjugate, whereas localization *in vitro* was demonstrated by indirect IF staining with the same combination of antisera. Connective tissue staining was demonstrated in kidneys, adrenals, ovaries, thyroid, spleen, lymph nodes, and liver. Under the conditions used, reactions *in vitro* were stronger than those *in vivo*. According to the authors, qualitative differences between reactions *in vivo* and *in vitro* are observed. Specific lesions have been reported only in the kidney, presumably because it is the most susceptible organ. The excellent reproducibility of nephrotoxic nephritis lesions has stimulated extensive studies. Ortega and Mellors (129) reported on fluorescent anti-globulin staining, histologic observations, and functional changes in nephrotoxic nephritis of rats induced by rabbit anti-rat kidney antibodies. Rabbit γ -globulin remained demonstrable for 3 months. Increase in rat γ -globulin which was demonstrable in rat glomeruli during the first postinjection week was ascribed to an accumulation of edema fluids, whereas further increases in glomerular concentration of rat γ -globulin during the 2nd to 15th week were attributed to the specific accumulation of autogenous rat antibodies to rabbit immune globulin. Similar observations are reported by Seegal (142). A delayed form of nephritis produced by injection of small amounts of duck anti-rabbit kidney antisera into rabbits was investigated. In contrast to the nephritis produced by large doses of nephrotoxic sera, no evidence of immediate renal damage was manifest. Duck antibodies were demonstrable in the kidneys of the injected rabbits for 7 to 15 days. With the development of signs of nephritis, rabbit antibodies become demonstrable.

III. IMMUNOFLUORESCENT STAINING OF MICROORGANISMS

An extensive bibliography of this subject may be found in Coons' recent review on the diagnostic applications of the method (29). The objectives

here are to review the most significant contributions that have been made since Coons' review of 1956 (24) and to discuss the literature in terms of the various ways in which immunofluorescence has been brought to bear on microbiological problems.

A. Viruses

Staining with labeled antibody lends itself to studies *in situ* of viral infections. The precise intracellular localization of viral antigen is demonstrable although the viral particles are submicroscopic. The method has been brought to bear at various levels of investigation: (a) in the initial characterization of viral agents as in measles (20, 51) and the foamy agent of monkey kidney cell cultures (13); (b) in the intracellular localization of known viral agents (9, 10, 12, 97, 124, 125); (c) as a potential diagnostic tool (18, 69, 70, 100); and (d) in more precise studies of virus-host cell interaction (8, 75, 98, 102, 133, 134, 154).

Enders' reports on the measles virus (50, 51) serve as a model of exploratory studies. A viral agent isolated in human kidney cell cultures was identified as the etiologic agent of measles by cytologic and serologic methods. Characteristic cytopathogenic changes in tissue culture were associated with virus by three serologic methods: (a) inhibition by human convalescent sera, (b) fixation of complement by the infected tissue culture and human convalescent sera, and (c) indirect IF staining of infected tissue with human convalescent sera. Enders reports cytoplasmic localization of the viral antigen. One of the basic precepts of immunology illustrated by this study is that all serologic reactions should be confirmed by the use of two or more test methods. Cohen *et al.* (20) arrived at similar conclusions independently. Viral agents were isolated on monkey kidney cell cultures from active cases of measles. They also demonstrated complement fixation and IF staining with infected tissue cultures and human convalescent sera. According to their report, the viral antigen was first demonstrable in the cytoplasm and later in the nucleus of infected cells. Baker *et al.* (3) later reported the observation of intracytoplasmic and intranuclear inclusions by both IF staining and electron microscopy.

Accurate quantitation of the number of infectious viral particles has been achieved for many groups of viruses by plaque count methods.

(A plaque is a discrete focus of infection in a susceptible cell population which may be produced by a single viral particle.) The method of demonstrating plaques depends on the virus-host cell system under consideration. Rapp *et al.* (134) adapted fluorescent antibody staining for plaque counts of measles virus in tissue culture. Infected monolayers of human epidermoid carcinoma cells grown on cover glasses were covered with methyl cellulose to prevent the spread of virus. Infectious foci were demonstrable by the indirect IF method after 3 to 6 days of infection under the conditions used. The number of foci was roughly proportional to the dilution of the virus suspension, according to these authors.

Eaton *et al.* (47) isolated a virus which he implicated as the etiologic agent of primary atypical pneumonia. This virus can be carried in chick embryos but produces no demonstrable pathology. The only test system available in his earlier studies was pathogenicity for the cotton rat. In a series of studies, Liu and Eaton (103), Liu (101), and Liu and Heyl (105) utilized immunofluorescent staining to investigate this system. Viral antigen was demonstrated by the indirect IF method with both convalescent sera from patients with primary atypical pneumonia and with rabbit antisera. The antigen was specifically localized in the bronchi of the chick embryo near the bifurcation of the trachea. The reaction of convalescent sera is of particular immunologic significance. It takes place only in the presence of complement-like activity of fresh normal serum. (Normal human and normal guinea pig sera were used by Liu.) This is, at present, the only reported IF staining reaction which is enhanced by complement, although undoubtedly others will come to light. Associated with primary atypical pneumonia is the development of cold agglutinins (antibodies which agglutinate the patient's own red cells at refrigeration temperatures) and agglutinins for streptococcus MG. Liu, Eaton, and Heyl (104) demonstrated that: (a) antibodies responsible for indirect staining of infected chick embryos were serologically unrelated to antibodies involved in cold agglutination or streptococcus MG agglutination; (b) such IF reactions are a more sensitive indicator of antibodies to primary atypical pneumonia virus than pathogenicity for the cotton rat; (c) convalescent sera usually show significant increases in antibody titer over acute

sera; and (d) IF staining reaction titers parallel titers of the neutralization test in cotton rats. Thus, labeled antibody staining is demonstrated to be specific for the virus. This is an example of a system in which IF staining is the only specific serologic *in vitro* test presently available. Still another example of the use of antibody staining in exploratory studies of viruses has arisen from observations on monkey kidney cell cultures. Laboratory workers were frequently hampered by "foamy" degeneration of kidney cell cultures. Carski (13) demonstrated, by direct IF staining, specific reactions of selected monkey sera with kidney cell cultures manifesting foamy degeneration. By use of indirect IF staining, he was able to determine which monkeys were free from infection with the "foamy agent" virus.

The elegant intracellular localization of viral antigens by immunofluorescence has attracted a number of workers to investigate known viruses by this method. Noyes and Watson (124) studied vaccinia viral infection in a tissue culture strain of human epidermoid cancer. Viral antigen was detected by direct fluorescent antibody staining. Specific staining was reported to occur in the form in cytoplasmic granules. Late in the infectious process, uniform staining extending into the nucleus was observed. Noyes (125) studied a neurotropic human virus, the Egypt 101 strain, with the aid of direct fluorescent antibody staining. The viral antigen was demonstrated in both infected mouse brain and in a tissue culture of human epidermoid cancer to which Noyes adapted the virus. Specific fluorescent antibody staining occurs only in the cytoplasm of infected cells, according to this report. Lebrun (97) studied the development of herpes simplex virus in tissue culture, chick embryo, and mouse brain by means of the fluorescent antibody method. In tissue cultures of human epidermoid carcinoma, viral antigen appears as intranuclear granules 24 hours after infection; 48 hours after infection, nuclear staining decreases and granular cytoplasmic staining appears. Buckley (11) observed labeled antibody reactions on tissue cultures infected with poliovirus. Similar localization of reactions was observed with types I, II, and III on several types of human cell cultures. The need for careful control of the dosage of virus in studies of the sequence of changes during the course of infection is brought out by Lebrun's report (98) on the poliovirus. When only a small proportion of the tissue

culture cells are infected with viruses initially, virus liberated from these cells infects other cells. Subsequent IF staining reactions occur with cells in different stages of the infectious process. However, by using 100 per cent initial infection, Lebrun obtained complete destruction of cells in 7 hours. Under these conditions, only cytoplasmic staining occurs up to the late stages of infection (5th to 7th hour). The late appearance of nuclear staining is ascribed to the development of nuclear lesions in degenerating cells.

Infection *in vivo* of wild and domestic rabbits with Shope papilloma was studied with fluorescein-labeled antibodies by Noyes and Mellors (126). Viral antigen could be detected only in a small proportion of the domestic rabbits and then only in the keratohyaline layers and in the keratinized layers, not in the proliferating cells or in the germinal cells. Specific fluorescent antibody staining was present only in the nuclei of infected cells. Viral antigen was more frequently detectable in wild cottontail rabbits. The authors postulate that a protein-deficient nucleic acid provirus which cannot be stained is present in the proliferating cells. Boyer, Denny, and Ginsberg (9) reported on careful studies of the development of adenovirus type 4 in HeLa cell cultures by indirect IF staining. Localization of virus was related to cytopathogenic changes as demonstrated by histologic methods and by electron microscopy. Conflicting results were obtained by cell fractionation. The discrepancy was clarified by careful fluorescent antibody studies.

Moulton (121) studied naturally occurring cases of canine distemper with the aid of direct IF staining. Hyperimmune rabbit sera served as a source of conjugate. Cerebella of infected dogs were stained. Specific staining was reported in nuclei of glial cells, whereas no specific staining occurred in demyelinated nerve fibers. Liu and Coffin (102) elucidated the pathogenesis of canine distemper viral infection by studies of the progressive changes in virus localization. Viral antigen was demonstrated with conjugates of globulins from convalescent dog sera. Ferrets were infected by intranasal inoculation. The virus invades the reticuloendothelial system via the cervical lymph nodes and progresses to the viscera, brain, and other organs of the body. Viral antigen appeared as intracytoplasmic granules. Coffin and Liu (18) propose a diagnostic procedure using stains of conjunctival smears.

The viral nature of Negri bodies in rabies was demonstrated with fluorescein-labeled antibodies by Goldwasser and Kissling (69, 70). The authors report specific staining of infected mouse brains with a human antiserum by direct and indirect IF staining and complement-staining procedures. The structures stained by the fluorescent antibody techniques were shown to correspond to Negri bodies by staining sections for visible light microscopy after examination of the fluorescent stain. Diagnosis of rabies by direct IF staining of salivary gland smears was attempted by Goldwasser *et al.* (70). Among 56 positive specimens on which detailed data are supplied, 49 were positive by culture of salivary gland smears. Of these 30 were positive by IF staining; 18 yielded occasional positive smears, and one was negative; a false positive reaction was reported in one of eight smears from one specimen. The authors report negative IF staining in 96 negative specimens.

Influenza virus has been subjected to extensive studies by the fluorescent antibody method (see Coons, 24, for a detailed review of the subject). Liu proposed a rapid diagnostic procedure for influenzal infections utilizing the fluorescent antibody method (100). Smears of cells from nasal washings are stained with fluorescein-labeled rabbit antiserum to influenza virus. The procedure is reported to be somewhat less sensitive than the demonstration of a rise of hemagglutination inhibition titer.

Immunofluorescence adds a new dimension to studies of virus-host cell interaction. Watson carried out IF staining studies of mixed infection (154). Chick embryos were infected with influenza and mumps viruses under varying conditions. Her results suggest simultaneous infections of cells with both viruses under certain conditions, though this is difficult to prove with antibodies labeled with a single fluorochrome.

Careful studies of phagocytosis of PR8 influenza virus *in vitro* were reported by Boand, Kempf, and Hanson (8). Virus in leukocytes was demonstrated by direct staining with reagents prepared from hyperimmune rabbit sera. Observations of staining reactions were confirmed by hemagglutination and chick cell agglutination reactions. Normal leukocytes in the presence of normal serum phagocytized no virus. Only surface adsorption was observed. Leukocytes from immune donors suspended in normal serum, as well as normal leukocytes suspended in im-

immune serum, phagocytized some virus. Optimal phagocytosis occurred with leukocytes from immune donors in the presence of immune serum. Phagocytosis *in vitro*, when observed, occurred within 30 minutes. These studies enabled Hanson, Kempf, and Boand (75) to undertake preliminary studies *in vivo* of virus phagocytosis. Mice treated by intraperitoneal injection with PR8 influenza virus were killed at time intervals up to 28 hours to demonstrate phagocytosis in peritoneal exudates. Some important differences from phagocytosis *in vitro* were reported. Leukocytes in normal mice and mice immunized with two injections exhibited phagocytosis after 16 hours but not after 8 hours, whereas leukocytes from mice immunized with three injections exhibited phagocytosis after 8 hours. This is in contrast to phagocytosis *in vitro* in 30 minutes. Further IF studies of phagocytosis *in vivo* and *in vitro* and its relation to immunity seem indicated.

Prince and Ginsberg (133) detected by IF staining a form of Newcastle disease virus in Ehrlich ascites tumor cells which was noninfectious, did not hemagglutinate, and did not fix complement. Infected cells were inoculated into mice. Noninfectious antigen was detected in harvested cells by indirect IF staining about three hours after infection. Staining appeared as cytoplasmic granules. One of the interpretations proposed by the writers is that incomplete viral antigen may be synthesized in the tumor cells.

B. Bacteria, Protozoa, and Fungi

Most reports on the application of IF staining to bacteriology deal with potential diagnostic uses of the method (41, 74, 86, 109, 118, 146-148). Also, the fate of injected bacterial antigens has been traced in experimental animals (35, 48, 89, 140, 141). A few observations on bacterial cytology have come to light in the course of studies of fluorescent antibody (42, 43, 148). Immunofluorescent staining has been used to advantage in relating the proportion of given varieties of bacteria on direct smears to the organisms isolated culturally (80).

A review of the literature on applications of the fluorescent antibody method to diagnostic bacteriology must be preceded by a discussion of some basic immunologic concepts. Immunofluorescent staining is a serologic test. Specificity and sensitivity are the principal factors to be considered in evaluating any serologic method.

Specificity is of prime importance. In diagnostic bacteriology, serologic tests with known antisera usually follow cultural isolation and identification. This limits the scope of cross reactions that must be considered. If crude mixtures of bacteria are used, a much wider range of cross reactions may come into play. The range of cross reactions with a given type of antiserum may vary with the method of testing. Thus the specificity of each new serologic test method must be evaluated; *i.e.*, extensive studies of cross reactions with various types of bacteria must be carried out. Similar specificity controls must be carried out for the Widal-Wassermann type of diagnostic test (*i.e.*, tests with known bacterial antigens and unknown patients' sera). The sera from patients with other clinically diagnosed diseases must be tested with the antigen by established test methods as well as by the proposed new methods.

The term "sensitivity" in the classical sense refers to the capacity of a serologic technique to detect the presence of antibodies. A technique which detects antibody reactions at higher serum dilutions than other methods or one which detects reactions not detectable by other methods is said to be the most sensitive. In my experience, the sensitivity of indirect IF staining (in terms of the highest positive serum dilutions) is usually approximately the same as complement fixation, greater than precipitation, and less than agglutination reactions. Some notable exceptions may be found, *e.g.*, nuclear autoantibody reactions. Estimates of the amount of antibody per milliliter of serum demonstrable by IF staining are not available at present. The capacity of a serologic test to detect small amounts of *antigen* is also of value in some situations. In this respect, IF staining is outstanding. Coons points out (24) that a single pneumococcus which contains about 5×10^{-11} mg of nitrogen can be readily detected. Small numbers of bacteria can be detected with a high degree of precision (120, 149). This becomes particularly valuable in dealing with organisms which cannot be cultured on artificial media (41).

Diagnostic use of fluorescent antibodies in the detection of enteric pathogens has been carefully investigated by Thomason and her associates at the United States Communicable Disease Center. The sensitivity of direct IF staining in the demonstration of antigenic components of *Salmonella* spp. is comparable to slide agglutina-

tion but less than the sensitivity of test tube agglutination according to Thomason, Cherry, and Moody (148). Similarly, Kabanova and Glubokina (86) in a study of unidentified *Shigella* species, reported greater sensitivity of agglutination reactions than of fluorescent staining. The problem is rendered even more difficult by the numerous cross reactions among the gram-negative enteric bacilli. Thomason, Cherry, and Edwards (146) report on a number of such cross reactions as observed by direct IF staining reactions. Thus stains of fecal smears with labeled antibodies to enteric pathogens yield unsatisfactory results (68 per cent of normal stool smears and 68 per cent of smears from typhoid carriers were positive with labeled typhoid antibodies). Several noteworthy cross reactions among the enterobacteriaceae are detailed by the authors. Thomason, Cherry, and Ewing (147) investigated the specificity of direct IF staining reactions with antisera to enteropathogenic *Escherichia coli*. Some cross reactions (three among 60 serotypes of *E. coli* and six among 102 serotypes of other enteric bacilli) were observed with nine labeled antisera. Thus, some false positive staining reactions would be expected with normal stools, although the number of organisms stained in specimens from cases of infantile diarrhea is much greater, according to the authors.

β -Hemolytic streptococci are classified into groups on the basis of precipitin reactions of the polysaccharides in the cell wall. Cross reactions between groups are not usually observed by precipitin tests. With fluorescent antibody staining, however, antibodies to group A polysaccharide react with group C β -hemolytic streptococci. Moody *et al.* (118) achieved complete specificity by adsorbing group A antisera with group C streptococci. Halpern *et al.* (74) stained throat smears for group A β -hemolytic streptococci. According to this report, 30 of 33 smears from culturally proven cases were positive, whereas none of 50 smears from negative cases reacted.

Erysipelothrix insidiosa, the causative agent of hog erysipelas, was subjected to some preliminary antibody staining studies. Dacres and Groth (39) observed no cross reactions by direct IF staining, whereas J. D. Marshall *et al.* (109) reported a cross reaction with *Bacterium anthracis*.

Indirect IF staining for *Treponema pallidum*

with patients' sera holds promise as a diagnostic tool (41). Antigens for conventional serologic tests for syphilis are mixtures of lecithin, cholesterol, and cardiolipids. These antigens are the product of several decades of careful research. At present there is a trend toward the development of tests utilizing *T. pallidum* as antigen. IF staining offers important advantages here; the method is usually at least as sensitive as complement fixation and requires less antigen. A few thousand bacteria per slide can easily be detected (14). Deacon, Falcon, and Harris (41) compared indirect IF staining for *T. pallidum* with *T. pallidum* complement fixation test, *T. pallidum* immobilization test, and a conventional type test (Venereal Disease Research Laboratory) with rabbit and human sera. Immunofluorescence and complement fixation tests were essentially of equal sensitivity in this study. Other test methods were less sensitive. The specificity of the method remains to be determined. The potential value of antibody staining for *T. pallidum* in the serodiagnosis of syphilis is born out by the reports of Olansky and McCormick (128) and Borel and Durel (8a). It seems likely that this will become the method of choice in the serodiagnosis of syphilis.

Most reports on the demonstration of protozoan parasites by immunofluorescence have come from Goldman's laboratories at the United States Communicable Disease Center. His earlier studies on *Endamoeba coli* and *Endamoeba histolytica* (64) were reviewed by Coons (24). More recently, Goldman studied *Toxoplasma gondii* by a method employing inhibition of staining (65, 66). A fluorescein conjugate was prepared from a human serum with a high methylene blue reduction titer for *T. gondii*. Sera containing antibody inhibited the specific IF staining of toxoplasmas. Carver and Goldman (15) described improved methods of preparing tissues infected with *T. gondii* for IF staining. Although the method holds promise as a diagnostic tool, the authors observed, "It would be unrealistic to believe that specific staining with fluorescent antibody can solve all problems of etiologic diagnosis in pathology when *Toxoplasma* organisms are suspected."

Preliminary studies on the specificity of direct IF staining reactions for *Candida albicans* are reported by Gordon (73). All strains of *C. albicans* could be specifically stained. In addition, all strains of *Candida tropicalis* and some strains

of other *Candida* species reacted with conjugates prepared from rabbit antisera to *C. albicans*. Also 5 of 46 strains of unrelated fungi stained with these conjugates. All the latter cross reactions and some of the cross reactions with other species of *Candida* were eliminated by adsorption with *Candida parakrusei*. The need for careful specificity controls on each serum is emphasized by the difference in the spectrum of cross reactions between the two reagents prepared from rabbit antisera to *C. albicans*.

The potential value of immunofluorescence in microbiology goes far beyond diagnostic applications. Some incidental observations suggest the value of the method in cytologic studies. The localization of flagellar and somatic antigens of *Salmonella* spp. is clearly illustrated in studies of Thomason, Cherry, and Moody (148). De Repentigny (42) demonstrated capsular reactions in pneumococci by direct IF staining. Similar observations had been made earlier by Coons *et al.* (30). De Repentigny and Frappier (43) also attempted to determine the location of the protective antigen of *Haemophilus pertussis* on the bacilli with labeled antibodies. The potential value of such studies is limited by the fact that bacteria are near the limit of resolution of the light microscope.

The studies of Hobson and Mann (80) illustrate another unique potential of immunofluorescent staining to relate organisms seen on direct smears with organisms isolated in pure culture from the same source. It is usually impossible to do this by other methods since morphology alone is inconclusive. Hobson and Mann describe methods for relating organisms isolated from the rumen content of cattle with organisms isolated in pure culture. As the authors point out, the method cannot be guaranteed to be entirely specific. However, by suitable adsorption and dilution procedures, cross reactions can be minimized.

The distribution of injected bacterial antigens has been subjected to close scrutiny by several investigators (35, 49, 89, 140, 141). Because of their medical significance, attention has been focused primarily on the antigens of β -hemolytic streptococci. In the studies of Schmidt (140, 141) protein and polysaccharide antigens were injected into mice. The distribution and persistence of these antigens in tissues were studied by direct IF staining. Carbohydrate antigens remained detectable for only 30 minutes after injection, whereas the M proteins were detectable for up

to 60 hours. Kaplan (89) reported the detection by indirect IF staining of M proteins of group A streptococci in the tissues of mice up to 8 days after injection. In both studies the antigen remained in the kidney for the longest period of time. The M protein of a type 12 strain of streptococci from an outbreak of pyelonephritis persisted in the kidney for the same length of time as other types of M proteins. These studies were carried out with mice treated by intraperitoneal injection with purified antigens. A somewhat different distribution of M protein antigen was observed in mice infected with group A β -hemolytic streptococci.

Localization of streptococcal hyaluronidase in tissue has aroused interest because antibodies to this enzyme are found in the serum of patients with the rheumatic fever and glomerular nephritis. The problem was investigated by injecting concentrated preparations of this enzyme intravenously into mice; frozen sections of mouse organs were stained with a fluorescein-labeled rabbit antiserum to the streptococcal hyaluronidase (Emmart *et al.*, 49). Staining reactions are reported to occur in all organs studied. Factors influencing the elimination of *Salmonella typhosa* endotoxin from the reticuloendothelial system were investigated by Cremer and Watson (35). Fatalities following parenteral administration of endotoxin to experimental animals were increased by x-ray irradiation, cortisone, thorotrast, and by two consecutive injections of the endotoxin. Cremer and Watson studied the tissue localization of the endotoxin by direct IF staining. Small rabbits were given intravenously 2 mg of the endotoxin. The authors report the finding of endotoxin in the reticuloendothelial system in lungs, liver, and spleen. After single injections, the endotoxin was largely eliminated in $\frac{1}{2}$ to 2 days. Cortisone or x-irradiation delayed the elimination of endotoxin, whereas thorotrast and double injections of the toxin markedly inhibited its elimination. The studies of Cremer and Watson deserve confirmation and extension.

IV. IMMUNOFLOUORESCENT STAINING OF TISSUE ANTIGENS

For the purpose of this review, fluorescent antibody studies of tissue antigens are classified into three groups on the basis of the types of antibody responses involved. *Heteroantibodies* as defined here are antibodies of one species developed against antigens of another species.

Isoantibodies react with tissue antigens of some members of the species of the antibody producer, but not with antigens of the antibody-producing individual. *Autoantibodies* react with tissue antigens of the antibody-producing individual.

A. *Heteroantibodies to Tissue Antigens*

The two major types of antigens considered in this category are (a) biochemically isolated tissue antigens and (b) the classic heterophile, species specific, and organ specific antigens.

Biochemically isolated products of tissue have been studied, of which hormones and muscle myosin are two prime examples (36, 52, 53, 84, 94, 96, 110, 150). In such studies, the fluorescent antibody method is used essentially as a histochemical tool. Although the method is potentially of unique specificity, *each* antiserum must be tested for cross reactions. Minor impurities may, and often do, stimulate antibody formation.

J. M. Marshall (110) was the first to report on this type of study. Using a modified direct IF staining procedure, he investigated the pituitary localization of antibodies to adrenocorticotrophic hormone (ACTH). Reactions were reported to be localized in the β cells of the anterior pituitary. As Coons indicates in his review (24) of Marshall's paper, further specificity controls of this reaction are indicated. More recently, Cruickshank and Currie (36) studied localization of pituitary hormones. Reactions of most antisera to human pituitary hormones occurred in the β cells of the anterior pituitary. However, staining was not abolished by adsorption with specific antisera. It is noteworthy that they demonstrated extensive cross reactions between antisera to human pituitary ACTH, growth hormone, and thyrotropic hormone preparations by precipitin tests. The identity of reactions was further demonstrated by adsorption experiments. Similarly, antisera to postmenopausal gonadotropin and thyrotropic hormone preparations extracted from urine cross reacted completely with each other but failed to cross react with hormone preparations made from pituitaries; *i.e.*, antisera to thyrotropic hormone preparations from pituitary and urine failed to cross react. These studies illustrate the need for careful specificity controls. Lacy and Davies (96) studied reactions of antisera to commercial preparations of insulin (from beef and pork) by direct IF staining. Islet cells of beef, cat, and mouse pancreas sections stained, whereas guinea pig, rabbit, and human islet cells

failed to stain, according to this report. Adsorption of labeled antibodies with pork insulin abolished staining, whereas adsorption with glucagon (a pancreatic hormone with insulin-antagonizing activity) failed to inhibit staining.

Rabbit antisera to myosin prepared from chick embryo skeletal muscle have been studied for several years in the laboratories of J. M. Marshall (52, 53, 84, 94, 150). Immunofluorescent reactions are localized in the A bands of skeletal muscle according to Finck, Holtzer, and Marshall's report (53). Holtzer, Marshall, and Finck (84) reported on studies of embryonic myogenesis. Myosin was demonstrable by IF staining before the development of histologically demonstrable bands. According to Finck and Holtzer's recent studies (52) of the specificity of antimyosin antibodies, no cross reactions are demonstrable with chick smooth muscle or other cell types studied. Klatzo, Horvath, and Emmart (94) reported on reactions of labeled antibodies to human myosin. Aggregation of specific staining was reported to occur in atrophic muscle fibers from cases of myotonic dystrophy.

Another example of the histochemical localization of a partially purified tissue antigen by IF staining is the study of Nairn, Fraser, and Chadwick (122). They attempted to localize hog renin in hog organs by direct staining. However, antibodies formed to impurities in the hog renin preparation. Although cross-reacting antibodies could be removed by adsorption, localization becomes quite difficult in such situations. Extensive studies on the tissue localization of γ -globulin and other serum proteins have been reported from the laboratories of Dixon (45), Mellors (116), Gitlin (61), and others. Some of these studies are considered with reports on antibody synthesis and reactions *in vivo*. An authoritative discussion of these papers may be found in the recent review by Mellors (113).

Heterophile, species specific, and organ specific antigens are identified on the basis of classic serologic tests. Heterophile antigens are found in tissues of many species. Species specific antigens are found in all organs of a given species, and organ specific antigens are found in a given organ of many species. Fluorescent antibody studies extend these concepts to the cellular or tissue component level. The classic example of a heterophile antigen is the Forssman antigen. Tanaka and Leduc (145) reported that Forssman antigen is found in the form of droplets in the

endothelium and adventitial connective tissues of blood vessels in all organs of guinea pigs, cats, dogs, mice, and chickens. The distribution of this antigen in other types of cells was reported to be variable in different species.

Species specific antibodies react with all organs of the given species. Most reports on IF staining with species specific antibodies have dealt with kidney antisera. Cruickshank and Hill (37) were the first to demonstrate the localization of species specific antibodies in the basement membranes of various organs. They used rabbit antisera to rat kidney and rat organs as a test system. Roberts (136) reported specific reactions of rabbit antisera to rat glomeruli with the lens capsule of the eye as well as with the lining of blood vessels. Hiramoto, Goldstein, and Pressman (78) studied rabbit antisera to two human tissue culture cell lines (HeLa and fetal liver). Stromal elements and connective tissues of various organs of the body reacted with these sera by indirect IF staining. Conversely, Goldstein, Hiramoto, and Pressman (68) stained two permanent cell lines, HeLa and 407 liver, with rabbit antisera to human thyroid and to human melanoma. These species specific reactions indicate that the cell lines are of connective tissue origin. Hiramoto, *et al.* (79) confirmed and extended the observations of Cruickshank and Hill (37). Species specific reactions were demonstrated in basement membranes, capsules, blood vessel walls, and other connective tissue elements. Localization *in vivo* and *in vitro* was similar, but the latter reactions were considerably stronger.

Hill and Cruickshank (76) also demonstrated kidney specific antibodies. A conjugate of an antiserum to whole kidney stained not only basement membranes of tubules, glomeruli, and blood vessels (species specific) but also cytoplasm of tubular epithelium (organ specific), whereas conjugates of antisera to rat glomeruli and lung stained only basement membranes and blood vessels. The kidney specific nature of the epithelial reaction was further demonstrated by adsorption experiments. Weiler (157) also demonstrated localization of kidney specific antibodies in the cytoplasm of tubular epithelium. Specificity of the reactions was adequately confirmed. In the earlier papers of Louis and his associates (93), the authors maintained that the reactions reported by Weiler (156) were non-immunologic in nature. This view was based on the histologic congruence of their nonimmuno-

logic reactions with the fluorescent antibody reactions reported by Weiler. However, such histologic coincidence of reactions does not necessarily prove their identity. It is noteworthy that Fisher and Fisher (54) failed to demonstrate kidney-localizing antibodies in the sera of dogs that had rejected homotransplanted kidneys.

Humphrey (85) demonstrated the origin of platelets by IF staining. Labeled rabbit antibodies to guinea pig platelets were used to stain guinea pig bone marrow and spleen smears. Only megakaryocytes stained specifically.

B. Isoantibodies

Several investigators have studied blood group isoantibodies by IF staining (1, 19, 63, 144, 159). However, only limited success has been achieved. Heteroantibodies to blood group substances were the first to yield positive fluorescent antibody reactions. The distribution of group A substance, H substance, and Lewis^a substance in human gastric mucosa was studied by Glyn, Holborow, and Johnson (63) by direct IF staining with conjugates derived from rabbit sera. Specific localization and differences in resistance to 90 per cent ethanol fixation in different sites are detailed by the authors. Alexander (1) reported on agglutination titers and IF staining reactions of several red cell-agglutinating systems. Negative results were reported with labeled isoantibodies, including human anti-A, although the conjugates had high agglutinating titers. Only certain rabbit antishrimp and rabbit antihuman A substance antisera yielded positive staining reactions.

Whitaker *et al.* (159) were the first to report the staining of red cells with anti-A and anti-B isoantibodies. They reported positive IF reactions only with cell suspensions pretreated with ethylenediaminetetraacetic acid (EDTA). Cohen, Zuelzer, and Evans (19) reported on further studies of red cells with isoantibodies. Strong positive staining with A and B isoantibodies was achieved by the direct IF method. Reactions of A and A₁ antibodies were distinguishable. Staining reactions were less sensitive than agglutination in detection of blood group isoantibodies. Direct staining, according to this report, was not sufficiently sensitive to detect Rh(D) isoantibodies. These were, however, demonstrable by indirect IF staining. The observed reactions were weak but specific. By these methods, the authors demonstrated fetal red blood cells in the maternal circulation during the last trimester of pregnancy.

This important report will undoubtedly be checked in many laboratories.

Szulman (144) studied the distribution of the A and B blood group antigens throughout the body. The specific antigens were found in three locations according to this report: in the cells and secretions of mucous secreting glands, in the stratified epithelium, and in the vascular endothelium.

According to a preliminary report of Danaher, Friou, and Finch (40), specific fluorescent antibody reactions are demonstrable with leukocyte isoantibodies by indirect IF staining. This is a difficult system to study because of the non-specific staining problems involved.

C. Autoantibodies

By definition, autoantibodies react with some tissue component(s) of the individual producing the antibody. They might be associated with autoimmune diseases. The criteria for establishing the autoimmune etiology of a disease are set forth in Witebsky's postulates (162): "(1) the direct demonstration of free, circulating antibodies that are active at body temperature or of cell-bound antibodies by indirect means; (2) the recognition of the specific antigen against which this antibody is directed; (3) the production of antibodies against the same antigen in experimental animals; (4) the appearance of pathological changes in the corresponding tissues of an actively sensitized experimental animal that are basically similar to those in the human disease." Immunofluorescence is a useful tool for demonstrating the autoantibody nature of serologic reactions, determining the specificity of the reaction, and characterizing the antigen.

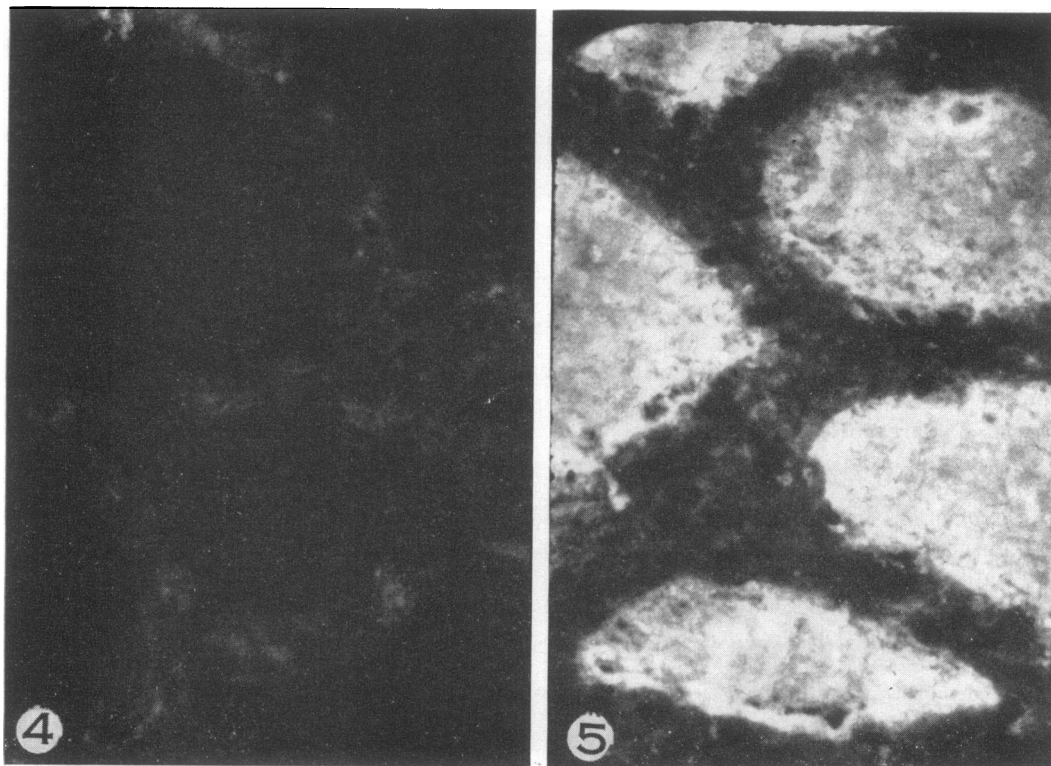
Chronic thyroiditis is the best documented example of an autoimmune disease (162). Several fluorescent antibody studies of this disease have been reported (6, 7, 77, 81, 161). Figures 4 and 5 illustrate the appearance of indirect IF staining reactions of a normal serum and a chronic thyroiditis serum on sections of a monkey thyroid.

White (161) reported localization of thyroiditis autoantibodies in the colloid of the thyroid and the glandular epithelium. He also reported on demonstrations of antibodies against thyroid extracts in the regional lymph nodes. Hiramoto, Engle, and Pressman (77) studied the localization of autoantibodies in the serum of a patient with chronic thyroiditis with the use of both fluores-

cein- and rhodamine-labeled antiglobulin antisera. They reported staining of the thyroid colloid and the edge of the glandular epithelium. Beutner *et al.* (7) studied the reactions of rabbit thyroid autoantibodies and rabbit spinal cord autoantibodies by both the direct and indirect fluorescent antibody method. The specificity of the reactions was demonstrated by cross reaction studies with rabbit thyroid and rabbit spinal cord sections. They observed staining of thyroid colloid and a thin rim of staining at the periphery of the glandular epithelium with rabbit thyroid autoantibodies. No cross reaction occurred with spinal cord autoantibodies. Further studies were carried out by the same group (6) on human, rabbit, and dog thyroid autoantiseria by the indirect staining method. They report limited cross reactions between the antisera and thyroids of other species. The reactions are predominantly species specific as well as organ specific. In all cases, staining reactions occurred in the thyroid colloid. The specificity of the reaction is amply documented by other serologic studies (162).

A fluorescent antibody spot test for thyroglobulin autoantibodies of thyroiditis patients was reported by Crawford, Wood, and Lessof (34). Fixed smears of human thyroglobulin are stained by the indirect IF method. Reactions are read with a Wood light, *i.e.*, a filtered ultraviolet light. The nucleohistone spot test of Friou (57) for antibodies of lupus erythematosus patients (see below) served as a specificity control. In the hands of these investigators (99) the spot test proved to be more sensitive than the passive cutaneous anaphylaxis test of Ovary *et al.* (131) although this has not been confirmed yet.

Holborrow *et al.* (81) reported another type of autoantibody reaction with the thyroid, a reaction with the glandular epithelium. Figures 6 and 7 illustrate the appearance of a control and the cellular reaction. It is reported to be associated with antibodies which fix complement with a microsome fraction of the thyroid. Antibodies to the cells are usually associated with the colloid antibodies of thyroiditis. This cellular staining reaction was observed independently in the writer's laboratory. The nature of the disease processes with which they are associated, if any, is not yet established. The experimental production of such autoantibodies has not as yet been reported. Studies of this reaction are complicated by the affinity of epithelial cells for nonspecific staining.

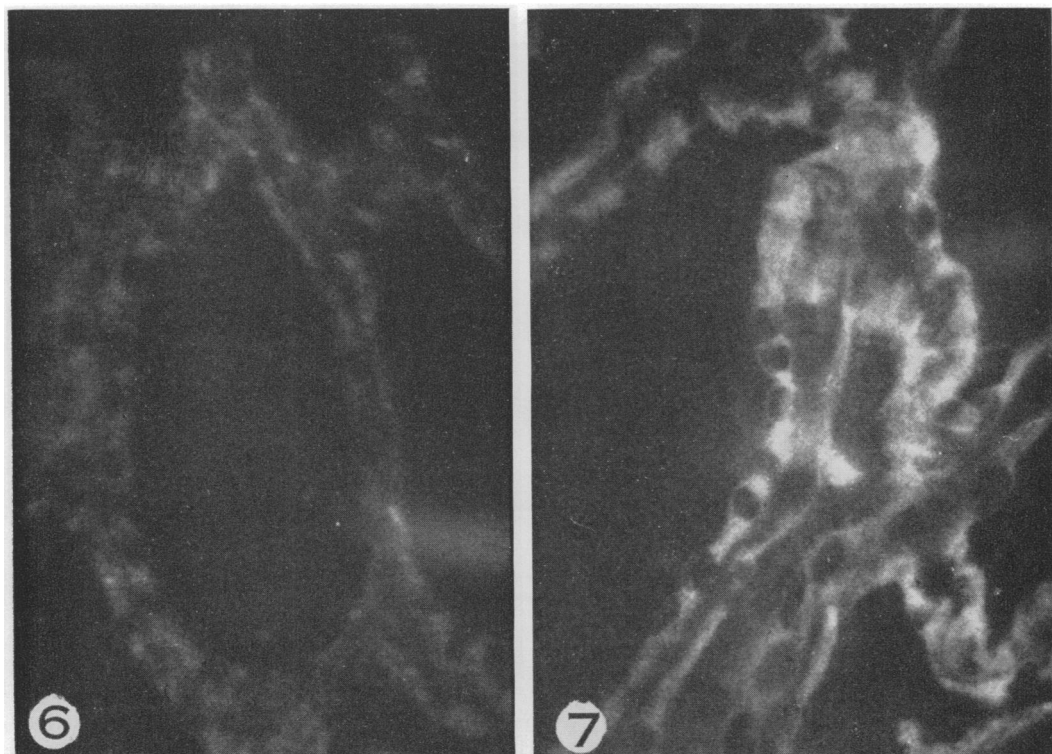


Figures 4 and 5. Indirect immunofluorescent staining of monkey thyroid sections with human sera diluted 1:10. *Figure 4.* Sections treated with a control serum (tuberculosis patient). This is a negative reaction. Slight nonspecific staining of cellular elements was observed in the original preparation. *Figure 5.* Section treated with serum of a thyroiditis patient. Immunofluorescent staining of the colloid is observed. This is an autoantibody reaction associated with thyroiditis (6, 161, E. Witebsky and E. H. Beutner, unpublished data).

Hargraves, Richmond, and Morton (75a) were the first to observe antinuclear antibodies in the sera of patients with lupus erythematosus (L.E.) (see Miescher, 117, for review and references). Friou, Finch, and Detre (58, 59) demonstrated nuclear staining with L.E. sera by the indirect IF procedure. This reaction occurred with all vertebrate cells studied by these authors. Only sera from L.E. cases and a few rheumatoid arthritis sera reacted, whereas all other sera tested were negative. These observations were quickly confirmed in other laboratories. Holborow, Weir, and Johnson (83) observed IF staining of nuclei with L.E. sera in several human tissues, including the thyroid. Figures 8 and 9 illustrate the appearance of the latter reaction. Bardawil *et al.* (4) observed nuclear reactions with L.E. sera by both direct and

indirect IF staining. These investigators reported inhibition of the reaction with the enzyme deoxyribonuclease. At approximately the same time Mellors, Ortega, and Holman (116) as well as Vazquez and Dixon (153) reported localization of human γ -globulin in the nuclei of leukocytes of L.E. patients. This was demonstrated by direct IF staining for human γ -globulin. γ -Globulin localization was substantiated, but the function or specificity of the localized γ -globulin could not be determined from these observations; *i.e.*, such staining reactions offer only presumptive evidence for the localization of some antibody of unknown specificity.

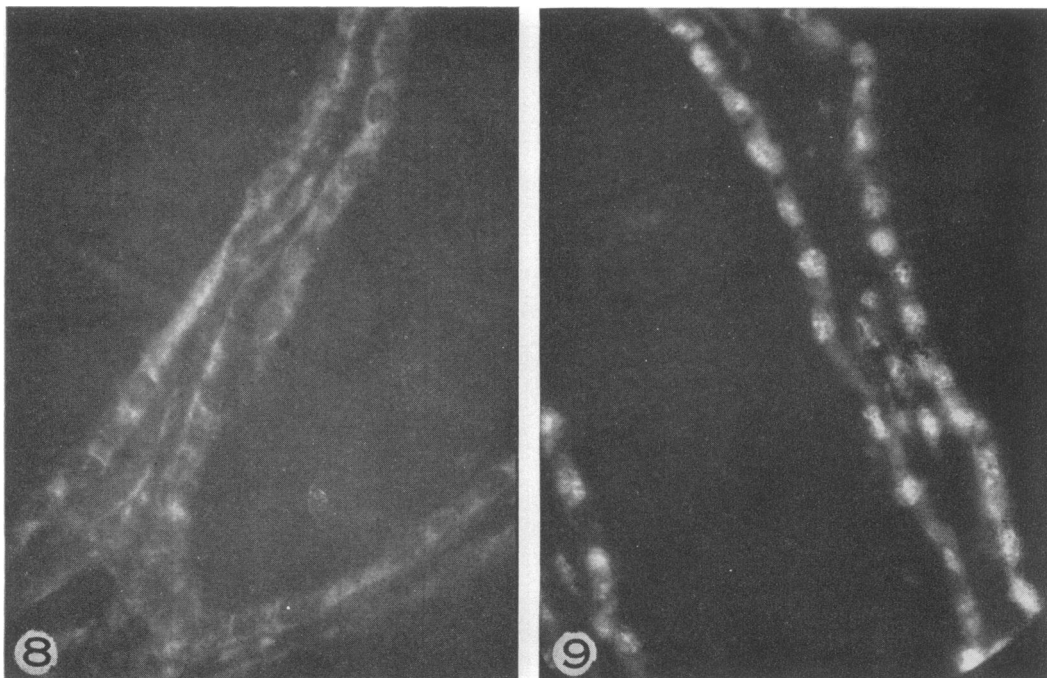
Friou (56) also reported that the extraction of cell smears with water (presumably distilled water) or 1 M NaCl removed staining reactions of the lupus sera tested, whereas extraction with



Figures 6 and 7. Sections of monkey thyroid stained as for figures 4 and 5 with human sera by the indirect immunofluorescent method. *Figure 6.* Negative reaction of a human serum. *Figure 7.* Immunofluorescent staining of the cytoplasm of the glandular epithelium obtained with serum from a patient with thyroid disease. This is an autoantibody reaction, distinct from the colloid reaction (see figures 4 and 5) but frequently associated with it (81, E. Witebsky and E. H. Beutner, *unpublished data*).

0.15 M NaCl did not. This he associated with the solubility characteristics of deoxyribonucleic acid (DNA) histones. He took further advantage of these solubility characteristics to prepare smears of DNA histones on slides for reactions with fluorescent antibody. Nucleohistone spots stained by the indirect method served as a screening test. Of 34 sera that were positive by IF staining, only 20 were positive by the L.E. cell test (phagocytosis of leukocytes *in vitro*). It is noteworthy that discrepancies were independent of titers as determined by IF staining. Recently Goodman *et al.* (72) reported on the failure of nuclear antibodies in the S 18 globulin fraction (see below) to give positive L.E. reactions. This may account for the lack of correlation between L.E. test results and IF staining reactions observed by Friou (55, 56). Crawford, Wood, and Lessof (34) report on the use of this

spot test. Also, Miescher and I have used it. Friou identified the antigen which participates in the nuclear staining reaction with antibody as nucleohistone. This contention is supported by the observation of Holborow and Weir (82). According to their report, nuclear staining is demonstrable in guinea pig tissues, including immature germ cells, but not in the heads of mature sperms. The authors point out that nucleohistones are transformed into protamine-like substances in the maturation of sperms. Some doubt is cast on the identity of the antigen by the observation that the enzyme deoxyribonuclease inhibits the reaction (4). It may be that IF staining antibodies are directed against the DNA-protein complex rather than the nucleohistones alone. Goodman *et al.* (72) fractionated sera from lupus patients on diethylaminoethyl cellulose columns. Ten fractions were tested for



Figures 8 and 9. Indirect IF staining of normal human thyroid sections with human sera as described for figures 4 and 5. Figure 8. A negative reaction with a tuberculous serum. Note the nonfluorescent nuclei. Figure 9. Section treated with the serum of patient with lupus erythematosus. Serologically specific staining of the nuclei is observed. This is an autoantibody reaction observed in all cases of lupus erythematosus and occasionally in related diseases (58, 59, 83).

total protein, γ -globulin, IF staining, tanned cell hemagglutination, and L.E. cell formation. Two active components were identified by both the tanned cell hemagglutination and IF staining. Goodman *et al.* regard these as the heavy (S 18) and light (S 6.6) globulins. Only the light globulin fraction was active in L.E. cell formation.

In brief, antinuclear autoantibodies have been demonstrated in the sera of patients with disseminated lupus erythematosus. The antigen is some part of the DNA-protein complex. However, autoantibodies which react with nuclei by staining, and lesions characteristic of the disease have not been produced in experimental animals. Thus the third and fourth postulates have not been fulfilled.

Kaplan (87) noted that rabbits immunized with beef heart infusion broth cultures of streptococci developed antibodies which react with heart tissue by the fluorescent antibody method. His careful studies of this phenomenon indicated that similar antibody responses were obtained in animals immunized with sterile broth, or with

beef heart, rat heart tissue, or human heart homogenates. Such antisera react with rabbit heart, including the heart of the rabbit producing the antibody. Staining reactions occurred between myofibrils and sarcoplasm, according to this report. They were paralleled by complement-fixing activity of the sera. The alcohol-soluble antigen involved in these immunohistological staining reactions is distinct from the cardiolipin antigen used in serologic tests for syphilis. Kaplan (88, 90) also demonstrated two types of autoantibodies to heart muscle in human sera after heart surgery and in sera of patients with rheumatic heart disease. Certain pathologic changes in heart muscle which appear after heart surgery may be associated with these autoantibodies. The first three of Witebsky's postulates are, at least in part, fulfilled by these outstanding studies.

Experimental allergic encephalomyelitis is induced by immunization with brain suspensions in Freund adjuvant. The disease is characterized by the development of paralysis, brain lesions,

and sometimes complement-fixing autoantibodies. The antigen with which the circulating antibodies react is in the alcohol-soluble fraction (163). Immunofluorescent staining with rabbit brain autoantibodies is localized in the myelin sheath of the white matter (7). Subsequent unpublished observations revealed staining in the gray matter. This is consistent with the view that submicroscopic deposits of myelin surround "unmyelinated" nerve fibers. In short, circulating antibodies react with an alcohol-soluble antigen in the myelin sheath. However, the nature of the encephalitogenic antigen(s) is a controversial subject (92). Furthermore, circulating antibodies play a minor role in the pathogenesis of allergic encephalomyelitis. Production of characteristic lesions by passive transfer of hypersensitive cells has been reported (132). If confirmed, the first of the Witebsky postulates would be fulfilled insofar as cellular hypersensitivity reactions are concerned. Thus far, cellular or delayed type hypersensitivity reactions have not been demonstrable by the fluorescent antibody method. Like other serologic methods, immunofluorescent staining reveals only circulating type antibodies.

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